Structural Revelations of TRAF2 Function in TNF Receptor Signaling Pathway

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Abstract

The tumor necrosis factor (TNF) receptor (TNFR) superfamily consists of over 20 type-I transmembrane proteins with conserved N-terminal cysteine-rich domains (CRDs) in the extracellular ligand binding region, which are specifically activated by the corresponding superfamily of TNF-like ligands. Members of this receptor superfamily have wide tissue distribution and play important roles in biological processes such as lymphoid and neuronal development, innate and adaptive immune response, and cellular homeostasis. A remarkable feature of the TNFR superfamily is the ability of these receptors to induce effects either for cell survival or apoptotic cell death. The downstream intracellular mediators of cell survival signal are a group of proteins known as TNFR associated factors (TRAFs). There are currently six canonical mammalian TRAFs. This review will focus on the unique structural features of TRAF2 protein and its role in cell survival signaling.

Identification of TNF and Its Role in Death and Survival Signaling

The tumor necrosis factor (TNF) receptor and ligand superfamily are widely distributed and are important for the proper function of the immune system. Currently, over 20 receptors have been identified including TNF-R1, TNF-R2, Fas, CD30 and CD40.[1] Agents that can manipulate the signaling of these receptors are currently being used and are showing promise towards the treatment and prevention of many human diseases.[2-4]

An interesting dichotomy of the TNFR superfamily is the ability of these receptors to induce both cell survival (proliferation and differentiation) and apoptotic cell death.[1,5,6] The fate of the cell depends on the intracellular region of TNFR members of the superfamily, specifically those with or without death domains (DD). Receptors that contain DD, such as Fas, DR4 and DR5, are mostly pro-apoptotic whereas receptors without DDs, such as TNF-R2, CD40, CD30, Ox40, 4-1BB, LTβR and TRANCE-R (also known as RANK), induce mostly survival effects. The functional divergence within the receptor superfamily is a consequence of the recruitment and assembly of different signaling proteins to the intracellular portion of the receptors (Fig. 1).

One of the most thoroughly studied member of the TNF-ligand superfamily is TNF-alpha. Many anecdotal but persuasive observations of tumor necrosis or regression by TNF-alpha were in cancer patients who had concurrent bacterial infections. Such stories have been noted throughout history and all over the world. In particular, pioneering clinicians in the late 19th century began treating various kinds of tumors including sarcomas, cancers of the bone and connective tissues, breast cancer, ovarian cancer, Hodgkin's disease, and melanoma by inducing acute skin infections,

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TRAFs, edited by Hao Wu.

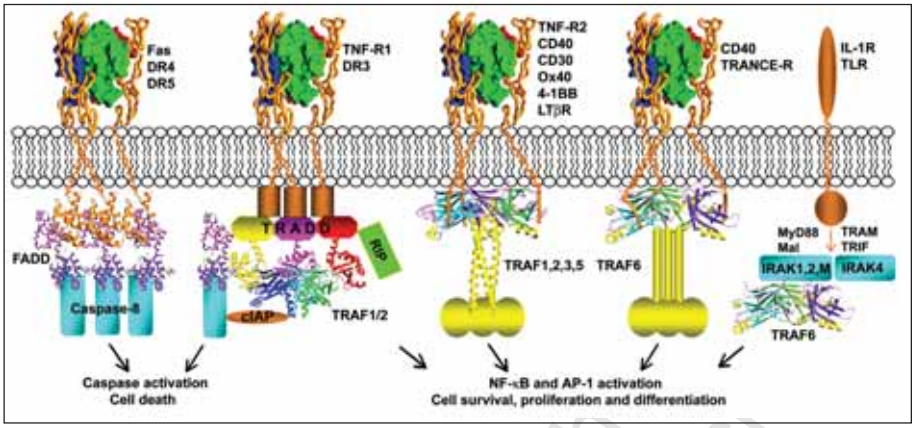


Figure 1. Intracellular signaling pathways for the TNFR superfamily and the IL-1R/TLR superfamily. Proteins with known structures are shown as ribbon drawings. Hypothetical transmembrane helices are built to connect extracellular and intracellular domains of these receptors (shown in orange). The amino terminal domains of TRAFs are shown as yellow spheres. Reproduced from Wu H.[44]

such as erysipelas.[7] The underlying mechanism of this novel "toxin" cancer therapy was attributed to a factor that could be produced and released by immune cells such as macrophages that have been stimulated by bacterial endotoxins.[8] The promise of TNF as a cancer cure led to the molecular identity of TNF through purification, characterization, and cloning.[9-12]

Further research with TNF for its anti-tumor activity led to the realization that TNF is a pleiotropic cytokine important in host defense against pathogens and capable of inducing cell survival, proliferation, and differentiation, as well as cell death, mediated by two TNF receptors, TNF-R1 and TNF-R2.[13-15]

Identification of TRAFs as Major Signal Transducers of the TNFR Superfamily

The TNFR superfamily members that promote survival signaling are those without DD in the intracellular region leading to the direct recruitment of adapter proteins called TNF receptor associated factors (TRAFs).[16-18] Currently, there are six canonical mammalian TRAFs (TRAF1-6) identified, of which all but TRAF4 are involved in the signal transduction of the TNFR superfamily,[16,19-29] and a recently identified "noncanonical" member, TRAF7.[28,29] Among the TRAF proteins, TRAF1, 2, 3 and 5 are considered TRAF2-like because they recognize and associate with TNF receptor family members through a conserved sequence motif on these receptors. In contrast, TRAF6 has a unique sequence requirement for its binding sites that does not overlap with TRAF2.[17] TRAF7 is also implicated in a map kinase signal transduction pathway, similar to the functions of other TRAFs.[29] However, not much is currently known about the upstream TRAF7 activation mechanism.

TRAF signaling activates transcription factors in the nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) family,[30,31] which can turn on numerous genes involved in cellular proliferation, differentiation, and regulation of immune response. Most TRAF proteins can be divided into two domains, the N- terminal RING and Zinc finger downstream signaling domain and the C-terminal TRAF domain. The TRAF domain can be further divided into a TRAF-N domain and a TRAF-C domain, which are important for self-association and receptor interaction, respectively.[16] TRAF7 does not conform to the canonical TRAF domain organization. It also consists of N-terminal RING and zinc finger domains, but instead of the TRAF domain, it consists of seven WD40 repeats.[29] Recent studies on the activation mechanism of TRAF2 downstream signal transduction has shown the involvement of a unique lysine-63 linked nondegradative polyubiquitination event, as shown by

the negative regulation of NF-κB activity by a TRAF2-interacting deubiquitination enzyme that was specific for nondegradative polyubiquitin chains.[32-34]

Interestingly, TNF-R1 and TNF-R1-like receptors possess the intrinsic capability to induce either cell death or cell survival. The mechanism by which these opposite cellular fates can coexist within one receptor lies on the recruitment of a multifunctional protein, TNF receptor-associated DD (TRADD), which can interact with both the DD within the receptor, as well as TRAF2.[35] The amino terminal domain of TRADD (TRADD-N) can recruit TRAF2,[35] while the carboxyl terminal DD of TRADD can recruit a death effector signaling protein called Fas associated DD protein (FADD) and a DD-containing Ser/Thr kinase called receptor-interacting protein (RIP), via DD-DD interactions.[35-37] Therefore, the fate of the cell depends on which proteins associate with TRADD, since both TRAF2 and RIP contribute to survival signaling,[38,39] whereas FADD recruitment activates caspases to induce apoptosis. However, the regulation of survival and death pathways from TNF-R1 is likely to be more complex and may involve cellular inhibitors of apoptosis (cIAPs), FLICE-inhibitory proteins (FLIPs) and c-Jun N-terminal kinase (JNK).[40-43]

Over the last six years, several TRAF protein structures, including TRAF2, TRAF3, and TRAF6 both alone and in complex with receptor peptides, have been determined[44] (Table 1). In addition, thermodynamic studies on TRAF2-receptor interactions were conducted and the results are summarized in Table 2. This chapter will focus on the structure and signaling mechanism of TRAF2. For detailed analyses of TRAF3 and TRAF6 structures, please refer to their respective chapters in the book.

Domain and Oligomeric Structures of TRAF2

Unique Anti-Parallel β-Sandwich Topology of TRAF2 C-Domain

The unique topology of the TRAF-C domain was first revealed from the crystal structure of the TRAF2 TRAF domain (Fig. 2A, B), alone and in complex with a receptor peptide from TNF-R2.[45] The main structural architecture of the TRAF-C domain features an eight-stranded anti-parallel β-sandwich. The first sheet of the anti-parallel β-sandwich consists of β1, β8, β5 and β6 strands and β2, β3, β4 and β7 strands make up the second sheet. The results from the Structure Classification Of Protein (SCOP) database[46] and the automatic structural similarity search engine, Dali program[47] showed that TRAF-C domain represents a novel fold for an eight stranded anti-parallel β-sandwich.

A more detailed inspection of the TRAF-C domain reveals additional structural features of the β strands. The β strands, β2 and β7 of the second sheet, present a bulge due to its highly twisted state. Preceding the β1 strand, there is a short stretch of residues (348-350) labeled β0, which runs parallel to β2, immediately after the β-bulge in this strand. The side chains of β0 residues partly cover one edge of the β-sandwich. Therefore, the twisting of β2 appears to play a structural role in the TRAF-C domain. The structure of the TRAF-C domain in complex with TNFR-2 peptide revealed that β7 strand contains the primary receptor peptide interaction site, thus the β-bulge and the twist in this strand may also play important structural and biological roles. A three-turn helix is present in the crossover connection between strands β1 and β2. Comparison and superposition of the 48 independent copies of the TRAF-C domain of TRAF2 in different crystal forms[45,48,49] showed that the structures are highly conserved with r.m.s.d of around 0.3-0.6 Å, with the exception of the flexible β7-β8 loop (up to 3-4Å in Cα distance). Structural comparison of TRAF-C domain structures in the absence and presence of receptor peptide interactions shows little conformational change, which indicates that its overall architecture is fairly rigid.

Sequence analysis of the TRAF-C domain showed that a diverse set of proteins with unrelated functions to TRAFs also possesses the TRAF-C domain. These proteins include meprins, a family of extracellular metalloproteases,[50] MUL, a protein involved in Mulibrey Nanism syndrome, USP7 (HAUSP), an ubiquitin protease, and SPOP, a POZ (poxvirus and zinc finger) domain-containing protein.[51] Because of its similarities with meprins, TRAF-C domain is also known in literature as meprin- and TRAF-homology (MATH) domain.[50]

A recent protein crystal structure of seven in absentia homolog (Siah), revealed that its substrate-binding domain (SBD) adopts an eight stranded anti-parallel β strand structure similar to

Table 1. Experimental Structures of TRAFs and their complexes

Protein	Binding Partner and Sequence[a]	Method	Resolution	#Protein, #Partner[b]	PDB Code, Reference
TRAF2 (327-501)			2.2Å	6	1CA4 [45]
TRAF2 (310-501)	TNF-R2 (420-428) QVPFSKEEC	Co-crystallization	2.3Å	6, 2	1CA9 [45]
TRAF2 (310-501)	CD40 (250-266) PVQETLHGCQPVTQEDG	Co-crystallization	2.7Å	3, 2	1CZZ [49]
TRAF2 (327-501)	CD40 (250-254) PVQET	Co-crystallization	2.0Å	8, 8	1D00 [49]
TRAF2 (327-501)	CD40 V251I mutant (249-254) YPIQET	Co-crystallization	2.0Å	8, 8	1QSC [48]
TRAF2 (327-501)	CD30 (576-583) MLSVEEEG	Co-crystallization	2.0Å	6, 3	1D01 [49]
TRAF2 (327-501)	Ox40 (262-266) PIQEE	Co-crystallization	2.0Å	6, 6	1D0A [49]
TRAF2 (327-501)	m4-1BB (231-236) GAAQEE	Co-crystallization	2.5Å	6, 5	1D0J [49]
TRAF2 (327-501)	LMP1 (204-210) PQQATDD	Co-crystallization	2.0Å	3, 2	1CZY [49]

Table continued on next page

Table 1. Continued

Protein	Binding Partner and Sequence[a]	Method	Resolution	#Protein, #Partner[b]	PDB Code, Reference
TRAF3 (341-568)			2.8Å	2	1FLK [78]
TRAF3 (341-568)	CD40 (247-266) TAAPVQETLHGCQPVTQEDG	Soaking	3.5Å	2, 2	1FLL [78]
TRAF3 (377-568)	TANK (178-195) SVPIQCTDKTDKQEALFK	Soaking	2.9Å	1, 1	1L0A [91]
TRAF3 (377-568)	TANK (171-191) IATDTQCSVPIQCTDKTDKQE	Soaking	3.5Å	1, 1	1KZZ [91]
TRAF3 (377-568)	LTbR (385-408) PYPIPEEGDPGPPGLSTPHQEDGK	Soaking	3.5Å	1, 1	1RF3 [92]
TRAF6 (346-504)			2.4Å	1	1LB4 [93]
TRAF6 (346-504)	CD40 (230-238) KQEPQEIDF	Co-crystallization	1.8Å	1,1	1LB6 [93]
TRAF6 (346-504)	TRANCE-R (342-349) QMPTEDEY	Co-crystallization	2.0Å	1,1	1LB5 [93]
TRAF2 (327-501)	TRADD-N (7-163)	Co-crystallization	2.0Å	1,1	1F3V [75]
TRADD-N (1-169)		NMR			1F2H [76]

[a] m: mouse; otherwise from human. [b] number of protein and partner molecules per crystallographic asymmetric unit. This table was taken from Wu H.[44]

Table 2. Thermodynamic characterizations of TRAF2-receptor interactions

TRAF	Receptor/Adapter and Sequence [a]	K_d [b]	ΔH	$-T\Delta S$	ΔC_P	Method [c]	Reference
TRAF2 (310-501)	CD30 (573-583) SDVMLSVEEEG	40 μM	-14.0±0.8 kcal/mol	8.03 kcal/mol	-245 cal/mol•K	ITC	Ye H et al[73]
	CD40 (250-266) PVQETLHGCQPVTQEDG	60 μM	-9.5±1.0 kcal/mol	3.87 kcal/mol	N.D.		
	OX40 (262-266) PIQEE	50 μM	-13.0±0.9 kcal/mol	7.22 kcal /mol	N.D.		
	TNF-R2 (420-428) QVPFSKEEC	0.5 μM	N.D.	N.D.	N.D.		
	m4-1BB (231-236) GAAQEE	1.0 μM	N.D.	N.D.	N.D.		
	LMP1 (204-210) PQQATDD	1.9 μM	N.D.	N.D.	N.D.		
TRAF2 (327-501)	TRADD (7-163)	7.8 μM	N.D.	N.D.	N.D.	SPR	Park YC et al[75]

[a] m: mouse; otherwise from human. [b] K_d: dissociation constant; DC_P: heat capacity change with temperature. [c] ITC: isothermal titration calorimetry; SPR: surface plasmon resonance. N.D.: Not determined.

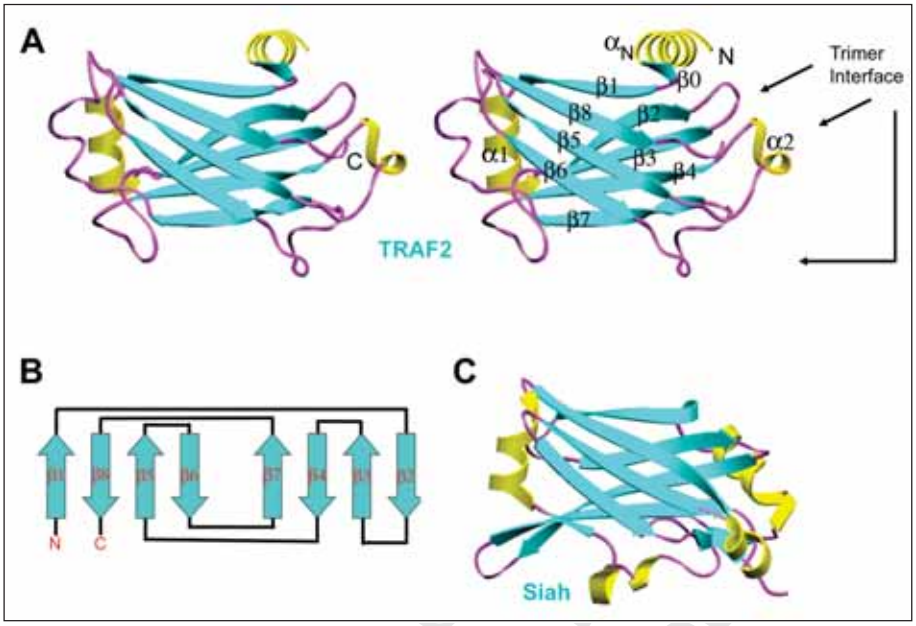


Figure 2. TRAF2 TRAF domain structure. A) Stereo drawing of the TRAF domain of TRAF2 with labeled secondary structures. B) Topology of TRAF-C domain. C) Ribbon drawing of Siah. Modified from Wu H.[44]

the TRAF-C domain structure, despite a lack of significant sequence homology[52] (Fig. 2B, C). In addition, the SBD is dimeric rather than a trimer. Interestingly, Siah is a member of the E3 ubiquitin ligase RING domain proteins and does have sequence similarity in this region to TRAFs. Furthermore, it appears that the SBD of Siah enhances TNF-mediated NF-κB activation, which suggests a potential functional similarity between Siah and TRAFs.

The Energetics and Specificity of the Trimeric TRAF Domain

The TRAF domain, consisting of the coiled-coil region and the TRAF-C anti-parallel β sandwich domain resembles the shape of a mushroom, in which the TRAF-C forms the cap and the coiled-coil region forms the stalk[45,48,49] (Fig. 3A, B). TRAF domain trimer portrays a perfect or near perfect three-fold symmetry. The diameter of the mushroom cap ranges between 50 to 80 Å while the stalk is approximately 50 Å long. The stalk consists of 5 characteristic coiled-coil heptad repeats (residues 311-347), which are seven amino acid residues denoted as abcdefg, in which the core residue positions of a and d are usually occupied by hydrophobic residues,[53] as is the case in this three-stranded parallel coiled coil structure. Both the coiled-coil domain and the TRAF-C domain contribute to TRAF domain trimerization.

The trimeric interface of the TRAF-C domain is formed by packing one end of the β-sandwich (the β2-β3, β4-β5 and β6-β7 connections) of one protomer against an edge and a face of the β-sandwich (β0, β1, and β8 strands, β5-β6 and β7-β8 connections) of the neighboring protomer (Fig. 3C). Both hydrophobic and hydrophilic residues are involved at the interface of the protomers, such as residues I355, Y386, A420, L421 and F491 of one protomer and K357, R385, R458, and D487 of the neighboring protomer.

The calculation of surface area burial upon TRAF domain trimerization reveals the importance of coiled-coil region in stabilizing the trimer formation. Roughly 640 $Å^2$ surface area is buried upon TRAF-C domain trimerization,[45] which is considered small compared to other stable protein-protein interactions.[54] This implies that the TRAF-C domain alone may not be sufficient for trimerization. In support of this analysis, biochemical studies on several TRAF domain constructs of TRAF2

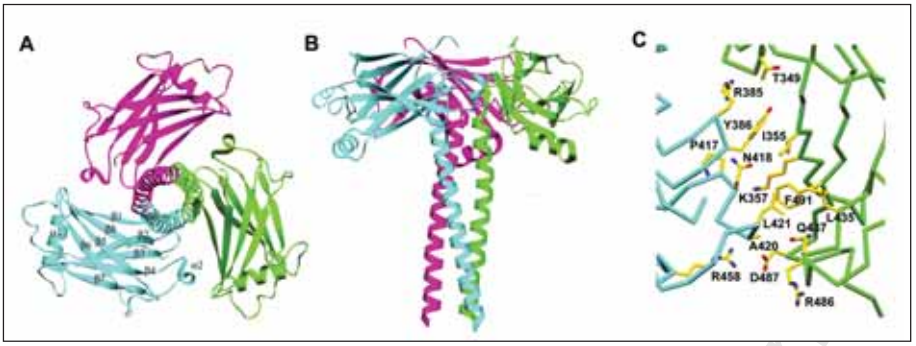


Figure 3. TRAF trimerization. A, B) Trimeric structure of the TRAF domain of TRAF2, shown with the threefold axis into the page and vertical, respectively. C) Detailed interaction between the TRAF-C domains in the trimer. Modified from Wu, H.[44]

showed that at minimum, three heptad repeats (residues 327-347) which increases the surface area burial to 1060 Å,^{2} are required for trimer formation.[45] The coiled-coil domain of TRAF2 appears to contain up to 14 heptad repeats, which could stretch to 140Å long and indicates a strong interaction.

Structural and computational analyses suggest that the major specificity determinant for TRAF domain trimerization lies in the TRAF-C domain residues. The analysis showed that the amino acid residues contributing to trimerization of the TRAF domain of TRAF2 are largely conserved among the TRAF family members.[45] This sequence conservation among the different TRAFs suggests that they may also be able to form similar homotrimers as well. On the other hand, the coiled-coil domains do not contain conserved sequences characteristic of trimeric coiled-coil, in fact TRAF2 coiled-coil was predicted by the Multi-coil program to form a dimeric rather than a trimeric coiled-coil.[55,56] Therefore, it appears that the TRAF-C domain, rather than the coiled coil domain, determines the observed specificity of TRAF trimerization, whereas the coiled coil is the major stability determinant for trimerization.

TRAF2-Receptor Interactions

Conserved Recognition of Diverse Receptors

The first glimpse of a TRAF2-receptor interaction provided by the crystal structure of the TRAF domain of TRAF2 in complex with a receptor peptide from TNF-R2[45] shows a different mode of interaction than that of TNF ligand to its receptor TNF-R1. Each peptide binds symmetrically to a shallow surface depression on the side of the mushroom-shaped trimer, extending from the top to the bottom rim of the mushroom cap (Fig. 4A, B). The peptide contacts only one protomer of the TRAF domain trimer. Therefore, this type of interaction does not rely structurally on TRAF2 trimerization, but relies energetically on avidity-mediated affinity enhancement for the receptor afforded by TRAF2 and receptor trimerization.

A major structural question is how TRAF2 can interact with a diverse group of receptors in the TNF receptor superfamily.[17] To go about answering this question, a total of eight crystal structures of the TRAF domain of TRAF2 in complex with several receptor peptides have been determined,[45,48,49] of which three structures are with CD40 receptor peptides (two are not shown) (Fig. 4C). These different complex structures include the three TRAF2 binding motifs proposed previously from biochemical and functional studies, the PxQx(T/S/D) (x = any amino acid) motif in LMP1, CD30, CD40, and CD27,[57-64] the ϕSxEE (ϕ=large hydrophobe) sequence in TNF-R2 and CD30,[16,59] and the QEE motif in 4-1BB and Ox40.[65]

Despite the high degree of sequence variability in the receptor peptides, the peptides contain a conserved binding mode at a common site on the TRAF domain. Superposition of seven different

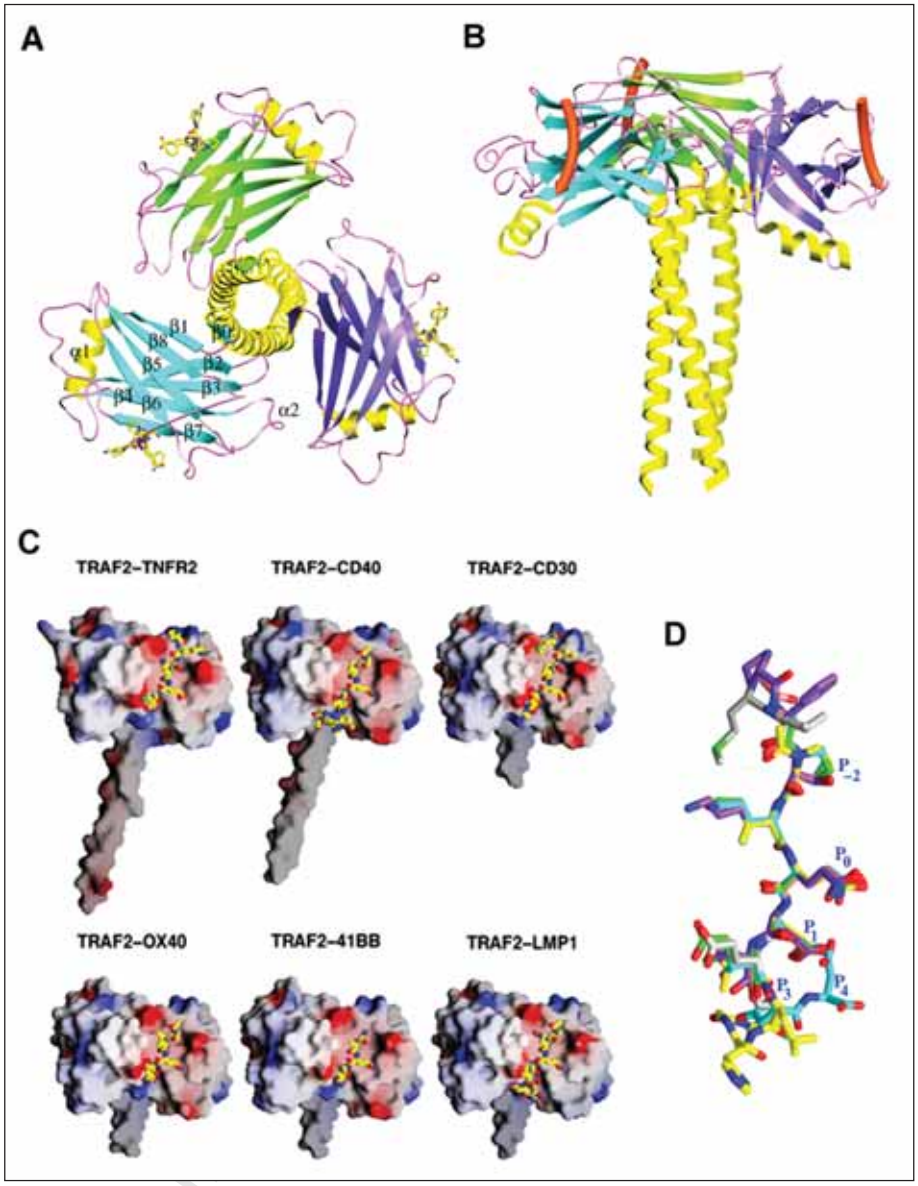


Figure 4. TRAF2-receptor interactions. A) Ribbon diagram of a TRAF2-receptor complex, looking down the threefold axis. The bound receptor chains are shown as stick models. B) Ribbon diagram of a TRAF2-receptor complex with the threefold axis vertical. The bound receptor chains are shown as arrows. C) Surface electrostatic representation of TRAF2-peptide complexes. D) Superposition of bound receptor peptides, showing the structural conservation of the main chain conformations and the side chain conformations at P_{-2}, P_0 and P_1 positions. Parts of this figure were modified from Ye H et al,[49] and Wu H.[44]

structures of receptor peptide complexes showed four highly conserved residues with r.m.s.d of less than 0.1Å along the main chain atoms of these residues (Fig. 4D). The third residue of this four residue core is invariably a Gln or a Glu. This position has the highest degree of conservation and is

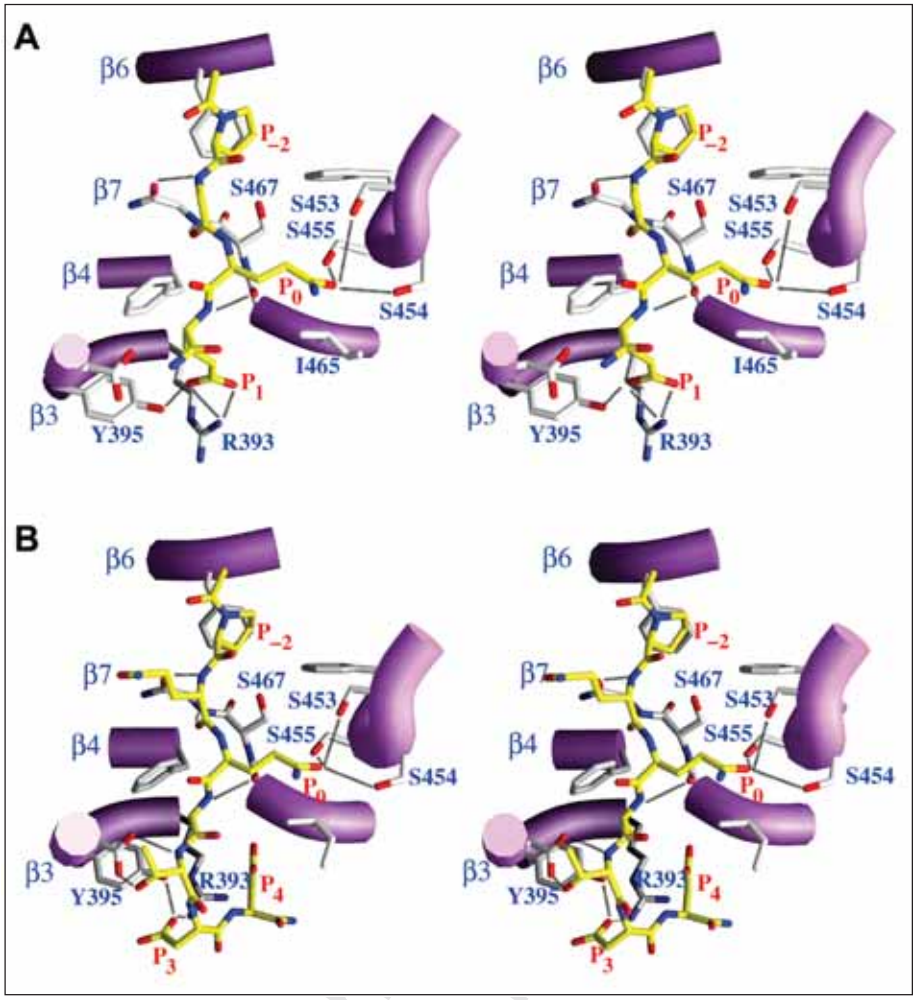


Figure 5. Detailed TRAF2-receptor interactions. A) Interactions seen in the major TRAF2-binding motif. B) Interactions seen in the minor TRAF2-binding motif. TRAF2 structures are shown as magenta worms and white stick models. The bound receptors are shown as yellow stick models. Modified from Ye H et al.[49]

denoted P_0 or the zero position of the TRAF binding motif. Hence, the labeling scheme for this four residue core is P_{-2}, P_{-1}, P_0, and P_1.

The receptor peptides are extended across four β-strands (β6 of the first sheet, β7, β4, and β3 of the second sheet) on one side of the β-sandwich structure of TRAF-C domain. Residues in position P_{-1} to P_1 runs anti-parallel and adjacent to the latter half of β7 strand (residues 466-468), which is immediately after the β-bulge. This leads to three anti-parallel β-edge main chain hydrogen bond formation between the peptide and the β7 of TRAF2 and creates an extra β-strand within the second sheet (Fig. 5A).

The formation of an extra β-strand by the extension of the peptide on the surface of a protein has been frequently observed in peptide-protein interactions.[66,67] Careful analysis of the peptide core position (P_{-2}, P_0, and P_1) revealed a highly twisted β-strand which can also qualify as a polyproline II (PPII) helix conformation structure. PPII structure has also been observed in peptide-protein

interactions such as in peptide recognition by SH3 domains[68] and class II MHC molecules.[69] The advantage of a PPII conformation in the peptides is that it maximizes side chain interactions with the protein surface. This is apparent in the peptide-TRAF2 structures where the twisting of the peptide allows for the side chains of P_{-2}, P_0, and P_1 to become buried at the TRAF2 interface. Therefore, the PPII conformation of the receptor peptides on TRAF2 maximizes both main chain and side chain interactions with the TRAF2 surface.

Key Residues of the Universal Major TRAF2 Binding Motif

The side chains of residues P_{-2}, P_0, and P_1 constitute the major structural determinant for peptide interaction with TRAF2 (Fig. 5A). The residues at these positions occupy distinct pockets within the TRAF2 surface. The P_{-2} residues are frequently Pro or Ser, which make extensive van der Waals contacts with TRAF2. In fact, the side chains of residues at P_{-2} are completely buried by the TRAF domain surface. In the case of Ser at P_{-2}, additional interaction is observed by hydrogen bond formation between the hydroxyl group and the side chain of S467 in TRAF2. The size and enclosure of P_{-2} binding pocket indicates only medium sized and nonpolar residues such as Thr, Cys, and Ile, can occupy this space. For example, residues such as Glu or Ala would not fit as well due to its charge and its small size, respectively. As predicted by the structural study of P_{-2} binding pocket, the Ala in P_{-2} position of 4-1BB receptor results in a weaker interaction with TRAF2, as evidenced by weaker binding affinity and electron density in this region of the complex structure.

The major structural determinants of Gln and Glu at P_0 position is the shape and hydrogen bonding interactions afforded by these particular residues. The aliphatic part of these residues pack against I485 while the hydrophilic region is surrounded by three hydroxyl groups of S453, S454, and S455 in TRAF2. Between the two residues, Gln is in the position to form hydrogen bonds with all three Ser residues of TRAF2, whereas Glu can only form one hydrogen bond. Due to the absence of charged residues near the vicinity of the P_0 site, there appears to be a need for the negative charge in Glu to be more heavily solvated than in Gln.

The P_1 position in most TRAF2 binding peptides is occupied by Glu. The carboxylate moiety of the Glu residue forms a bi-dentate ion-pair interaction with the side chain guanidinium group of R393 and a hydrogen bond with Y395 in TRAF2. The size of the P_1 binding pocket predicts a substitution with a smaller residue such as Asp residue, will not be sufficient to form the hydrogen bond that is observed with Glu.

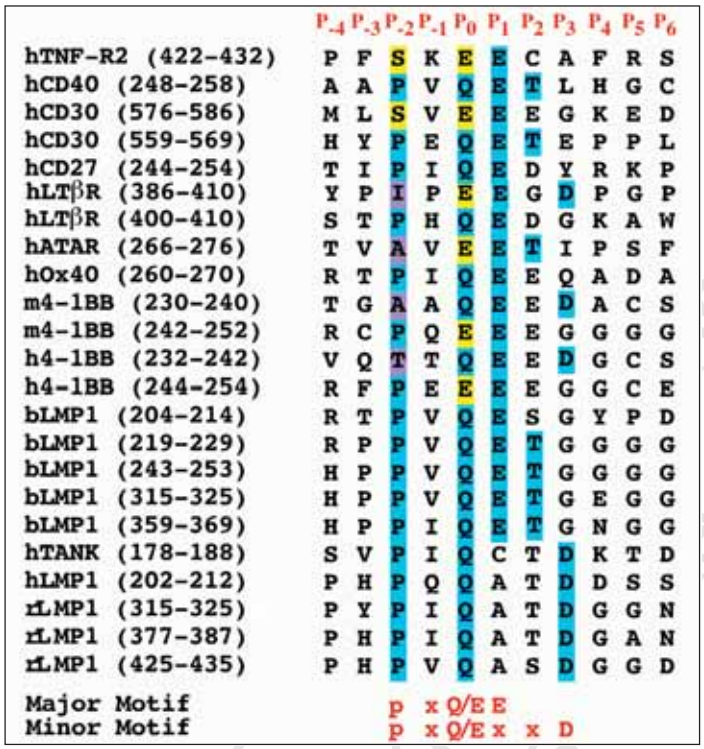
The sequence and structural conservations at the P_{-2}, P_0, and P_1 positions define the major TRAF2 binding motif. These positions are occupied by the consensus sequence px(Q/E)E, where proline is in lower case because it can be substituted by other medium size nonpolar residues (Fig. 6). The major TRAF2 binding motif can also be found on receptors which interact with TRAF1, 3, and 5, which explains the overlapping receptor-binding specificity of these TRAFs.

The Minor TRAF2 Binding Motif

The crystal structure of TRAF2 with LMP1[49] revealed a second TRAF2 binding motif that utilizes the residue at P_3 position rather than at P_1. The P_1 position is occupied by Ala in LMP1 and cannot make the same interactions as a Glu residue (Fig. 5B). However, the Asp residue of LMP1 at P_3 makes the same ion-pair hydrogen bonds with R393 and Y395 that the Glu makes at the P_1 of the major TRAF2 binding motif. This structural information along with sequence analysis shows the existence of a minor TRAF2 binding motif, px(Q/E)xxD (Fig. 6). In addition to LMP1, the intracellular protein, TANK (also known as I-TRAF)[70,71] possesses the minor TRAF2 binding consensus motif (Fig. 6) and may interact with TRAFs similarly as seen in the TRAF2-LMP1 complex.

Extent and Variations of TRAF2 Binding Motif

The next highest degree of structural conservation outside of P_{-2} to P_1 lies at P_2 and P_{-3} positions of TRAF2 interacting receptor peptides. Beyond P_2 and P_{-3} positions, there are large conformational differences among the various peptides (Fig. 4D). Therefore, the TRAF2 binding motif can incorporate up to 6 residues (P_{-3} to P_2). However, it should be noted that additional N- and C-terminal contacts are made with the TRAF domain by the receptors. For example, the TNFR-2 peptide-TRAF2



	P_{-4}	P_{-3}	P_{-2}	P_{-1}	P_0	P_1	P_2	P_3	P_4	P_5	P_6
hTNF-R2 (422-432)	P	F	S	K	E	E	C	A	F	R	S
hCD40 (248-258)	A	A	P	V	Q	E	T	L	H	G	C
hCD30 (576-586)	M	L	S	V	E	E	E	G	K	E	D
hCD30 (559-569)	H	Y	P	E	Q	E	T	E	P	P	L
hCD27 (244-254)	T	I	P	I	Q	E	D	Y	R	K	P
hLTβR (386-410)	Y	P	I	P	E	E	G	D	P	G	P
hLTβR (400-410)	S	T	P	H	Q	E	D	G	K	A	W
hATAR (266-276)	T	V	A	V	E	E	T	I	P	S	F
hOx40 (260-270)	R	T	P	I	Q	E	E	Q	A	D	A
m4-1BB (230-240)	T	G	A	A	Q	E	E	D	A	C	S
m4-1BB (242-252)	R	C	P	Q	E	E	E	G	G	G	G
h4-1BB (232-242)	V	Q	T	T	Q	E	E	D	G	C	S
h4-1BB (244-254)	R	F	P	E	E	E	E	G	G	C	E
bLMP1 (204-214)	R	T	P	V	Q	E	S	G	Y	P	D
bLMP1 (219-229)	R	P	P	V	Q	E	T	G	G	G	G
bLMP1 (243-253)	H	P	P	V	Q	E	T	G	G	G	G
bLMP1 (315-325)	H	P	P	V	Q	E	T	G	E	G	G
bLMP1 (359-369)	H	P	P	I	Q	E	T	G	N	G	G
hTANK (178-188)	S	V	P	I	Q	C	T	D	K	T	D
hLMP1 (202-212)	P	H	P	Q	Q	A	T	D	D	S	S
rLMP1 (315-325)	P	Y	P	I	Q	A	T	D	G	G	N
rLMP1 (377-387)	P	H	P	I	Q	A	T	D	G	A	N
rLMP1 (425-435)	P	H	P	V	Q	A	S	D	G	G	D
Major Motif			p	x	Q/E	E					
Minor Motif			p	x	Q/E	x	x	D			

Figure 6. Sequence alignment of TRAF2 binding sequences, illustrating the two TRAF2-binding motifs. h: human; m: mouse; b: bovine; r: rat. Modified from Ye H et al.[49]

interaction shows ordered residues starting at P_{-4} position and the CD40-TRAF2 structure shows ordered residues up to P_6. Incorporating these ordered residues may indicate that a complete TRAF2 binding sequence may contain up to eleven residues (P_{-4} to P_6). These additional residues outside of the core binding region are most likely exposed on the surface of the TRAF domain, which makes them tolerant to substitutions by other amino acids. As a final note, the actual TRAF binding region may vary from receptor to receptor since the conformations of end residues appear highly dependent on their side chain chemistry.

It should be pointed out that the presence of these motifs is often necessary but may not be sufficient for the receptor-TRAF2 interactions, and that other residues at different positions may also be important. For example, the P_2 residue may also contribute to TRAF2-receptor interaction since it is in close proximity to D399 of TRAF2 to allow hydrogen bond formations. In the CD40 receptor peptide, the P_2 is occupied by a Thr and the mutation of this residue to all but Ser eliminated association with TRAF2.[72] Therefore, it may not be too uncommon to find variations in the TRAF2 binding motifs.

Thermodynamics of TRAF-Receptor Interactions

Weak Affinity and Avidity

Several quantitative studies using isothermal titration calorimetry (ITC) and surface plasma resonance (SPR) on receptor peptide interactions with TRAF2 showed weak affinities between receptor peptides and TRAF in the absence of ligand. For example, the dissociation constants of CD40, CD30, and Ox40 peptides with TRAF2 range between 40-60μM, and 0.5 to 1.9mM for TNFR-2, 4-1BB, and LMP1 (Table 2).[73] These quantitative measurements of receptor peptides to TRAF2

likely represent the interaction of TRAF2 with actual full length intracellular receptor tails, since structural studies showed that only four core residues within receptor peptides act as the major determinant for TRAF2 interaction.[45,49]

The measured binding affinities for TRAF2-receptor interaction is relatively lower than most observed protein-protein and protein-peptide interactions.[67] This observation indicates that TRAF recruitment is entirely dependent on affinity enhancement through avidity by receptor trimerization. The exact magnitude of affinity enhancement is difficult to quantify and most likely depends on the conformational state of the trimerized or oligomerized receptors.

Favorable Enthalpy, Unfavorable Entropy and Induced Fit

ITC experiments on TRAF2-receptor peptide interactions consistently showed favorable enthalpy gain and unfavorable entropy loss, which indicate that these interactions are energetically driven by an exothermic mechanism. The enthalpy of TRAF2-receptor peptide interaction showed a large negative linear dependence with increase in temperature, as measured for TRAF2-CD30 interaction at 10, 20, and 30˚C.[49] This enthalpy dependence on temperature is indicative of specific interactions, rather than nonspecific, as shown from other thermodynamic studies involving protein-DNA interactions.[74]

The observed unfavorable entropy despite the burial of significant hydrophobic surfaces upon peptide binding is likely due to conformational restraints on the receptor peptide by TRAF2 interaction. Secondary structure prediction of the intracellular domains of most TNFR superfamily members shows a lack of preformed well-ordered three-dimensional structures. Therefore, this suggests that conformational changes and induced fit occur between TRAF2 and receptors.

TRAF2-TRADD Interaction: A Novel Mode of TRAF Signaling

The TRADD-N Domain

The interaction between TRAF2 and TRADD occurs through the TRAF domain of TRAF2 and the N-terminal domain of TRADD (TRADD-N). The structure of TRADD-N domain shows a α-β sandwich fold with a four-stranded anti-parallel β-sheet and six α helices[75,76] (Fig. 7A). There are two helices involved at each crossover between β-strands, β1-β2 (helices A and B) and β3-β4 (helices C and D). A hairpin-like turn is formed between β2-β3 strands. The remaining E and F helices are near the carboxy-terminus of the domain. The EF loop partially covers the exposed face of the β-sheet.

The α-β sandwich of TRADD-N is most similar to the family of ferredoxin-like α-β sandwiches.[77] Similar α-β sandwich topology has been observed in the structures of the palm domain of polymerases and the dimerization domain of carboxypeptidases. However, the extra helices in the β1-β2 and β3-β4 connections as well as the additional E and F helices makes TRADD-N a more elaborate structure.

Interactions and Energetics at the TRADD-TRAF2 Interface

The trimeric structure of the TRAF domain enforces the threefold symmetry to the stoichiometrically bound TRADD-N (Fig. 7B, C). The side view of the TRADD-TRAF2 complex shows TRADD bound to the upper rim of the mushroom cap, which adds a wing-like appearance to the complex structure. The carboxyl terminus projects upwards towards the membrane bound receptor direction. The orientation allows TRADD to interact with TNFR1 via the death domains and acts as a platform for other proteins to associate, such as TRAF2, FADD, and RIP (Fig. 7D).

The TRADD-TRAF2 interface partially overlaps with the site of TRAF2-receptor interaction. This indicates a competitive nature of TRAF2-TRADD and TRAF2-receptor interactions. Each TRADD-N molecule contacts one protomer of TRAF2, much like the receptor peptides. The interaction buries a surface area of 1500Å,^{2} which leads to small conformational changes in the Cα positions of TRAF2 (0.5-1.0Å) within or immediately adjacent to the TRADD binding site.

The interface between TRADD-TRAF2 resembles a “ridge into groove” type of contacts, exemplified by complementary elevations and depressions on the surfaces of TRADD-N and TRAF2

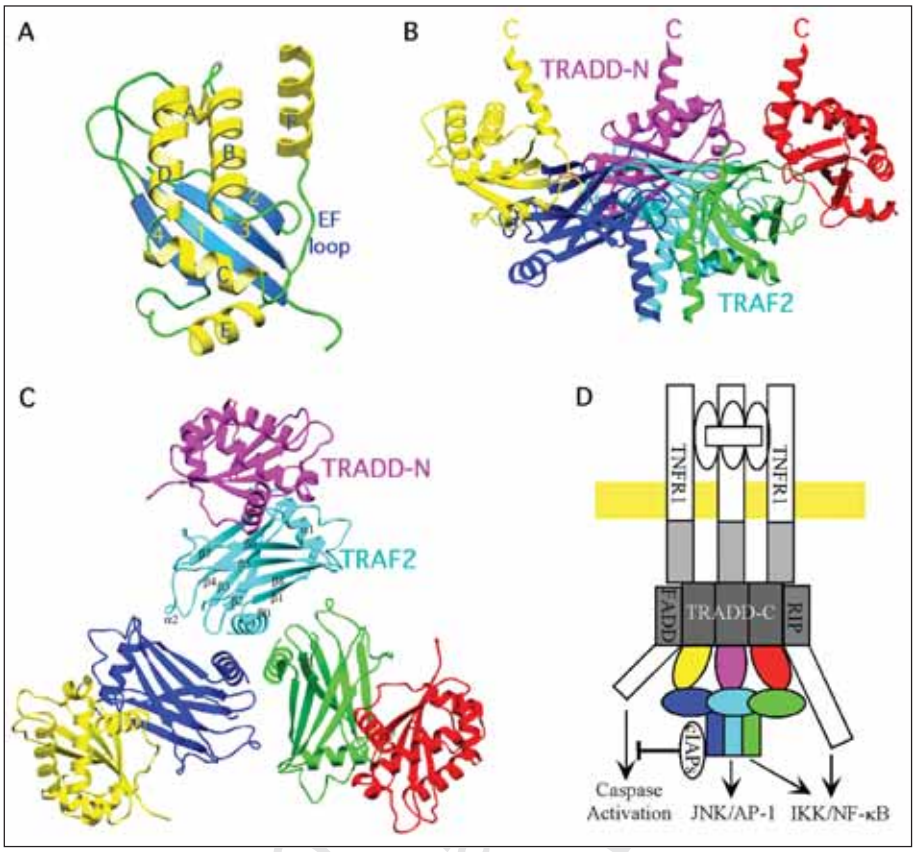


Figure 7. TRAF2-TRADD interaction. A) Ribbon drawing of the TRADD-N domain. B, C) Ribbon diagrams of the TRAF2-TRADD complex. D) Schematic representation of the TNF-R1 signaling complex. Reproduced from Park YC et al.[75]

TRAF domain (Fig. 8A). The interface can be separated into two distinct and adjacent regions (Fig. 8B). Region I consists of the shallow β-sheet face of TRADD-N and a surface protrusion of TRAF2 β7 strand, the following loop, and the loop between β3 and β4 strands. The specific residues that contributes to the TRADD-TRAF2 interaction in Region I are, Y16, F18, H65, S67, and I72 of TRADD and T401, H406, L471, and P474 of TRAF2 (Fig. 8C). The interaction Region II consists of a highly charged ridge formed by TRADD residues 143-149 in the EF loop and a surface depression formed between TRAF2 β6 and the following loop. Many hydrogen bond interactions are made by the residues in Region II, including anti-parallel main chain hydrogen bonds between the TRADD residues 145-147 and 448-450 of TRAF2, as well as side chain hydrogen bonds and salt bridges between R146 of TRADD with D445 of TRAF2, between R76 of TRADD and D450 of TRAF2, between Q143 of TRADD and S454 of TRAF2, and between D145 of TRADD with the main chain of G468 of TRAF2 (Fig. 8D). Many water molecules are also present at the Region II and at the boundary between the two regions (Fig. 8E).

Mutational studies of residues involved in TRADD-TRAF2 interactions in both Region I and Region II showed differential effects on the binding affinity irrespective of the surface area burial contributions. Alanine substitutions of residues in Region I (Y16, F18, H65, and S67 of TRADD) had a much more detrimental effect on binding affinity than residues in Region II. This result

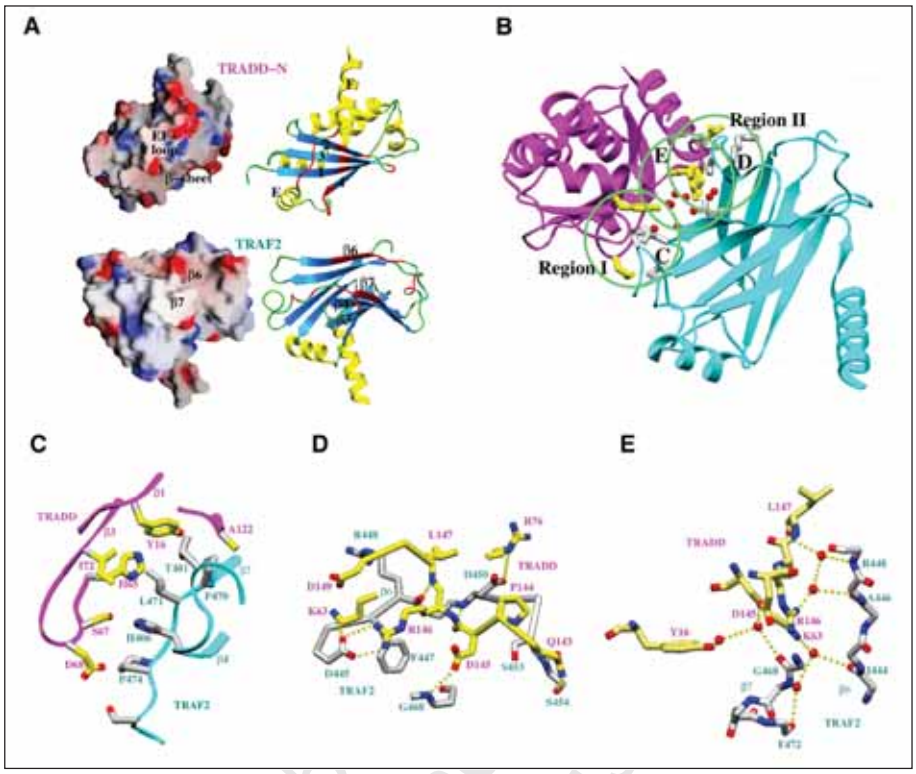


Figure 8. Detailed TRAF2-TRADD interaction. A) Interaction surfaces and their locations on the individual structures (in red). B) Molecular interactions at the two regions of the interactions. C, D, E) Details of region I, region II and water-mediated interactions, respectively. Modified from Park YC et al.[75]

indicates that despite the larger surface area burial of Region II compared to Region I, the largely hydrophobic interaction in Region I plays the dominant role in the energetics of the interaction.

Higher Affinity and Distinct Specificity of TRADD-TRAF2 Interaction

Surface plasma resonance experiments on TRAF2-TRADD interaction revealed a higher binding affinity (K_d = 7.8μM) compared to TRAF2-receptor interactions (K_d = 40μM-1.9mM).[73] The higher affinity between TRADD-TRAF2 suggests that TRADD may be a stronger inducer of TRAF2 signaling. This hypothesis was examined in cells expressing exclusively TNF-R1, which signals through TRADD, and cells that only expressed TNF-R2, which signals through direct TRAF2 recruitment. The strength of TRAF2 recruitment was measured by the activation of a major TRAF2 downstream effector, JNK protein kinase.[38] As predicted from the in vitro binding affinity studies, the JNK activation was much stronger for TNF-R1 than for TNF-R2 expressing cells.

The TRADD interaction with TRAF proteins appear to be limited to only TRAF2 and TRAF1 (Table 2). This selectivity by TRADD is not observed by TNF superfamily receptors lacking the intracellular death domain, since these receptors show similar binding specificities for TRAF1, 2, 3, and 5.[45,49,78] The ability of TRADD to associate with both TRAF1 and TRAF2 may have significance in the prevention of apoptosis by TNF-R1 activation (Fig. 9). Rothe et al has shown in TNF-R2 signaling complex, both TRAF1 and TRAF2 are constitutively associated with cellular inhibitors of apoptosis proteins (cIAPs), cIAP1 and cIAP2, and that this association requires the presence of both TRAF1 and TRAF2.[79] Therefore, as a consequence of the specificity of TRADD for TRAF1 and

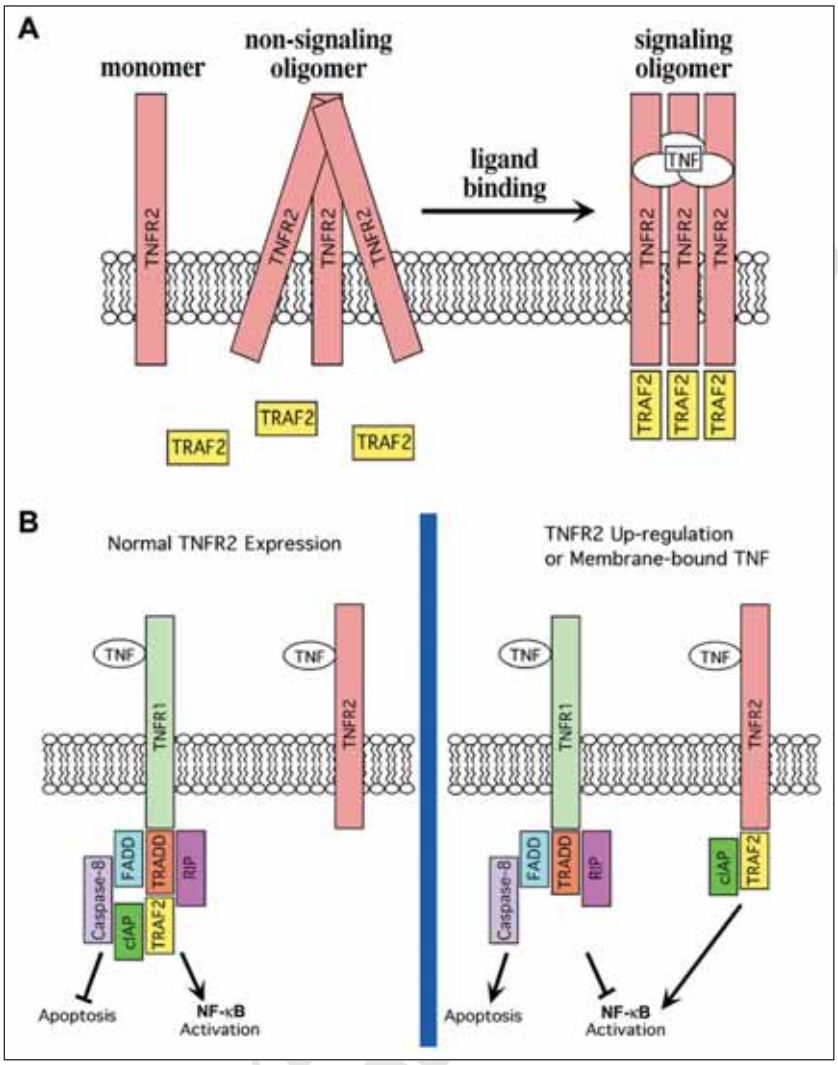


Figure 9. Principles of post-receptor signal transduction. A) Receptor activation and TRAF recruitment. B) Competitive TRAF recruitments and regulation of cell survival and death. Reproduced from Wu H.[44]

TRAF2, the cIAPs are brought to TNF-R1 and likely play an important role in blocking the apoptosis pathway.[40]

The predominant outcome of TNF-R1 activation is not apoptosis, as is the case for Fas receptor activation, but rather cell survival or proliferation. The evidence that TRADD binds specifically and selectively to TRAF1 and TRAF2 strongly supports the survival phenomenon. TRAF2 signaling has been shown in TRAF2 knockout studies to protect cells from apoptosis induced by TNF.[38] In addition, mutational studies on TRADD which resulted in reduced affinity for TRAF2 greatly sensitized cells to cell death.[75] These observations implicate TRAF2 as a critical determinant of cellular survival in the TNF-R1 pathway.

Based on these observations, a natural question arises as to when or in what situation does TNF-R1 activation lead to apoptosis? One possible answer may be through the mitochondrial release of Smac

protein through JNK activation.[43] Smac may interact with cIAPs and remove them from TRAF1 and TRAF2. Another possible answer may lie on the NF-κB-inducible protein c-FLIP. In the absence of NF-κB activation and c-FLIP, TNF-R1 can induce cell death through a cytoplasmic complex containing TRADD, RIP1, FADD, and caspase-8 activation.[42]

Summary: Emerging Principles of Post-Receptor Signal Transduction

Increased Affinity Through Avidity

Structural and biophysical studies on TRAF2-receptor and TRAF2-TRADD interactions showed that receptor peptides and TRADD contact one protomer of the TRAF domain trimer and that they interact with TRAF2 at low affinity. This suggests that receptor oligomerization and affinity enhancement through avidity is required for TRAF recruitment (Fig. 9). However, because a wide range of affinities between TRAF2 and receptors or TRADD have been observed (Table 2), the issue of whether different receptors would require different avidity contributions for TRAF2 recruitment is raised.

Interestingly, many TNF-like cytokine ligands, including TNF, are membrane-bound and therefore may be able to create a higher order of receptor aggregation through membrane-patching or clustering. This membrane receptor aggregation would then increase avidity and thereby enhance affinity for TRAF2. In support of this avidity induced affinity hypothesis, both soluble forms of CD40L and TNF have been shown to be weak inducers of TRAF2 signaling via CD40 and TNF-R2, respectively.[80,81] However, this is not the case for TNF-R1 activation by soluble TNF due to a much stronger TRADD-TRAF2 interaction and recruitment to the receptor.

Based on the TRAF2-receptor structures and the biophysical measurements of binding affinities, the need for receptor aggregation for efficient TRAF2 signaling corresponds well to what is evident in biology. Many other members of the TNF receptor superfamily such as CD30, Ox40, and 4-1BB ligands are membrane bound and mediate signaling in this state. The soluble ligand forms of these TNF receptor superfamily members are reported to be inefficient in activating the intracellular signal transduction pathway. In fact, such soluble ligands have been implicated in the role as decoys to down-regulate receptor activity.[82,83]

Competition Based Regulation of Survival and Death by TRAF2

TRAF2 plays a central role in the regulation of cell death and cell survival by TNF receptors, TNF-R1 and TNF-R2. Studies have shown that overexpression of the survival receptor TNF-R2 sensitizes cells to TNF induced apoptosis.[84-88] This contradictory outcome can be explained by the competitive recruitment hypothesis (Fig. 9). It may be that abundant TNF-R2 levels on the cell membrane draws all the TRAF2 as well as TRAF1 to its intracellular domain, which then depletes cIAPs from TNF-R1 to block caspase activation. Thus, cell survival or death is dependent on intracellular pool of cIAPs associated with TRAF1 and TRAF2.

Similar type of TNF-R1 and TNF-R2 interplay may exist among the other members of the TNF receptor superfamily, such as CD40, CD30, LTβR and CD27. These receptors have also shown the ability to induce apoptosis in certain circumstances.[89] Activation of any of these receptors could lead to sequestration and or degradation of TRAF1, TRAF2, and cIAP proteins.[90] This will then make the cells vulnerable to TNF induced apoptosis via TNF-R1 signaling.

Remaining Questions

Significant amount of structural information on TRAF2 interaction with receptor peptides and TRADD-N has provided an elegant explanation and agreement with biological observations of TRAF2 function. However, many questions are left unanswered regarding the molecular mechanism of TRAF2 signaling. For example, is TRAF2 in monomeric or in a constitutive trimeric state before recruitment to receptors? Is the activation of downstream effectors dependent on oligomerization or on conformational changes induced by receptor interaction? What is the exact molecular basis for this activation? Finally, the ultimate challenge will be in translating the structural and

functional studies into potential therapies for many important diseases involving TNF receptor superfamily members.

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